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ANIMAL MODELS FOR THE STUDY OF DRUGS IN ISCHEMIC STROKE

S. E. Karpiak, A. Tagliavia, and C. G. Wakade

Department of Psychiatry, College of Physicians and Surgeons, Columbia University, and the Division of Neuroscience, New York State Psychiatric Institute, 722 West 168th Street, New York, New York 10032

INTRODUCTION

During the last decade there has been an increased recognition and detailing of central nervous system plasticity. More recently neuroscientists have described those cellular, membrane, and biochemical events that characterize brain injury processes, including central nervous system stroke. These parallel developments in neurobiology have created a climate in which the neuroscientist now actively explores the heretofore unexplored possibility that one can effectively treat brain injuries or pathologies by transplantation or pharmacological intervention.

Does the inherent plasticity of the mammalian central nervous system provide a foundation for efficacious pharmacological intervention as a treatment for brain injury due to stroke? The need to test this hypothesis is clear since stroke is this nation's third leading cause of death (1). The functional losses sustained by the surviving stroke victim enact massive hospital, rehabilitative, and emotional costs. Medical research has made considerable progress in identifying factors that may assist in preventing its occurrence. Now, neurobiology has focused on determining what types of intervention may significantly aid in reducing the extent of central nervous system damage and thereby functional impairment that result after stroke. This increased effort and optimism is based upon research which has now identified certain pathological and biochemical events that characterize stroke. "Positron emission tomographic studies have demonstrated regions of low cerebral blood flow and preserved oxygen metabolism after cerebral infarction (1, 2), suggesting that strategies aimed at increasing perfusion (hemodilution or the

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administration of pentoxifylline, prostayclin, tissue plasminogen activator, or calcium-channel blockers) may salvage threatened tissue (3). On the other hand, animal models of global ischemia and recent data from autopsy studies performed after cardiac arrest in humans have demonstrated progression of neuronal damage for at least 72 hr, despite rapid reperfusion (4, 5). These findings suggest that therapy should be aimed at correcting abnormal ion fluxes and the release of damaging cytotoxic by-products of lipid metabolism (such as free radicals) known to be triggered by even brief periods of ischemia (6, 7); thus, calcium-channel blockers, barbiturates, or naloxone may be appropriate." (1).

For the basic laboratory neuroscientist, the task of "screening" pharmacological agents for stroke treatment is dependent upon the use of animal models. The choice of an animal model is affected by many factors (available personnel, costs, end-point measurements, etc). However, as with any animal model for a human disorder, a primary criterion for choice should be how closely the model approximates the clinical condition.

Stroke can refer to a number of pathological conditions. Certain neurological deficits characterize the clinical syndrome of stroke; there is an acute onset of these symptoms which persist for more than 24 hr and have vascular origins limited to (*a*) thrombotic or embolic occlusions; (*b*) spontaneous rupture of a vessel that results in hemorrhage. Excluded are occlusions or rupture of vessels due to neoplastic, traumatic, or infection processes that cause a vascular pathology (8). The vast majority (greater than 80%) of all diagnosed strokes fall into the category of thrombotic or embolic (arterial occlusion) occlusion that results in infarction (8). The central nervous system infarct is characterized by the loss of oxygen and glucose due to a deprivation of blood supply. The next most frequent type of central nervous system infarct is due to either a subarachnoid or intracerebral hemorrhage. Based upon this frequency, hemorrhagic models/studies may be less appropriate for investigation. In addition, some are based upon vascular "reperfusion" for which there is no apparent parallel clinical condition. Therapeutic regimens for ischemic versus hemorrhagic strokes probably will be quite different, especially since the mortality due to hemorrhagic stroke is greater than 80% and the mortality for ischemic stroke is 25% (8).

A frequently used "stroke" model is whole-animal anoxia or hypoxia. However, since the entire organism (all organ systems) is deprived of oxygen, such approaches do not specifically address the pathology of central nervous system stroke. Complicating factors which arise from resulting organ dysfunctions and those interactions with the central nervous system make treatment strategies very different, as compared to an ischemic event localized only to the central nervous system.

Since a preponderance of strokes are ischemic (i.e. arterial occlusion) (8),

we describe animal models which meet that criterion. These animal models involve occlusion(s) of the vascular supply to the central nervous system, and include the elimination or reduction of central nervous system blood supply to (*a*) entire cerebral hemisphere(s) (i.e. global ischemia); (*b*) focal regions of the brain; (*c*) multiple loci in the brain. Characteristics of the models are detailed by including surgical procedures, pathology, resulting dysfunctions, and their relevance to the human condition.

GLOBAL ISCHEMIA

Bilateral Hemispheric Ischemia

BILATERAL LIGATION OF THE COMMON CAROTID ARTERY Reducing or eliminating blood supply to both cerebral hemispheres characterizes global ischemia that leads to massive cerebral infarction and swelling (edema). The ease of executing these models of global ischemia makes them attractive for basic research studies. One apporach is to use extrinsic transcutaneous compression of the neck with an inflatable cuff (9). The procedure is adaptable to most mammalian species, and was probably one of the first models of ischemia studied in the rat (10). More often, this technique has been used to study reversible ischemia, thereby determining at what time point the compression leads to death, or assessing when each cellular and functional pathology occurs in the cerebral hemispheres. The relevance of this approach is questionable because it has no parallel in the human condition other than strangulation or surgical manipulation. In addition, the procedure results in compression of both arterial as well as venous blood supply. Human global ischemia is typically a consequence of myocardial infarction, hypotension or ventricular fibrillation (11).

Bilateral occlusion of the common carotid arteries is the simplest and most direct approach for inducing global ischemia (10). This is achieved by isolation of the common carotid artery through an incision on the ventral surface of the neck. The salivary glands are moved laterally, and the carotid sheath exposed. Both the vagus and sympathetic nerves are separated from the common carotid artery, which is then permanently ligated.

In the rat, with permanent bilateral ligations of the common carotid artery, there is almost a 64% mortality 24 hr later. Variables can include: temperature, sex, strain, size, diet, surgical technique (10, 12–16). The causes of mortality are largely brain swelling (edema) and focal lesions (infarcts). Swiftness, the ability to use large numbers of animals, ease of identifying brain swelling, and the use of mortality as an independent measure make this an appealing model system. In addition, the extracranial surgical approach eliminates invasion of the CSF compartment, surgical damage to central nervous system tissue, or mechanical disruption of the blood brain barrier.

OCCLUSION OF THE VERTEBROBASILAR ARTERIAL SYSTEM Recognizing that a temporary (less than 1 hr) occlusion of the common carotid artery alone does not lead to a consistent cerebral infarct, Pulsinelli (17) proposed a model that involves temporary bilateral common carotid artery occlusion together with permanent bilateral occlusion (electrocauterization) of the vertebral arteries in the rat. This four vessel occlusion model is advantageous as common carotid artery occlusions (temporary) occur without anesthesia. The vertebral artery coagulation is done first under anesthesia. Because coagulation of the vertebral arteries is done stereotaxically, the EEG (via implantation of electrodes into the calvarium) of rats must be monitored during the common carotid artery occlusion. If the EEG is isoelectric ("flat"), then the surgical occlusion of the vertebral arteries is considered successful. The occurrence of bilateral vertebral artery occlusion in man is rare and the prognosis for this type of ischemia is generally good (18).

Unilateral Hemispheric Ischemia

Unilateral hemispheric global ischemia can be accomplished in the Mongolian gerbil because most (not all) have an incomplete circle of Willis, wherein there is little or no communicating vascular system between the hemispheres (19–23). Following either a unilateral partial or complete ligation of the common carotid artery, ischemia is largely lateralized to the ipsilateral cerebral hemisphere in the majority of the animals (45–50%). This is advantageous, because the animal can be used as a type of control, allowing comparisons between the ligated and nonligated hemispheres. In those that survive the permanent unilateral common carotid artery occlusion there can be a wide variability in the extent, if any, of the resulting cerebral infarct. This can present serious methodological problems when analyzing certain injury parameters (22), but can be minimized by the use of temporary ligations. The gerbil is not particularly amenable to quantitative functional (neurological and behavioral) testing.

For global ischemia in the gerbil, the locus of the primary infarct is initially in the posterior portion of the hemisphere. Anterior areas become ischemic with time, indicative of the spread of ischemic damage (peri-infarct zones). Although the model implies that there is no vascular circulation to the ischemic hemisphere, there is cross circulation from the intact carotid to the anterior portion of the hemisphere (the anterior cerebral arteries join and enter the interhemispheric tissue as a single vessel) (24–29). Therefore, the primary infarct (following unilateral common carotid artery occlusion) is indeed due to a lack of posterior communicating arteries, and the "global" aspect of the infarct is a function of the spread of ischemic damage into anterior hemispheric regions that do have blood supply. Further, part of the damage results from direct compression (not lack of vasculature) by the greatly enlarged ischemic

hemisphere. Accordingly, there is considerable evidence of partial blood flow to the "infarcted cerebral hemisphere". The damage is not exclusively ischemic.

This model is again attractive because of its simplicity, although its applicability to the human condition is debatable. Although the gerbils are not ideally suited for neurological testing, motor deficits can be observed (30). A large number of gerbils (30–35%) will develop either focal or generalized seizures (29). This pathology complicates interpretation of results. As a result of the variability of this lack of vascular communication occurring between the hemispheres, larger numbers of animals are needed to effectively complete pharmacological studies (22).

FOCAL ISCHEMIA

The most commonly occurring stroke in humans is due to an occlusion(s) within the arterial system innervating the central nervous system. Animal models have employed various approaches toward highly localized occlusions at various sites in the arterial system of the brain (31).

Occlusion of the Anterior Cerebral Artery

Although the clinical occurrence of anterior cerebral artery occlusion is uncommon (31), when it does occur in man the resulting symptoms include: contralateral hemiplegia, particularly of the leg, reduced grasping reflex, urinary incontinence, emotional dysfunction, malaise, and spastic paralysis. Critchley (32), using case reports and pathological material from humans, largely verifies this symptomatology. Campbell & Foster (1944) (33), 2–3 weeks after occluding the anterior cerebral artery in the monkey (*Macaca mulatta*), report neurological deficits comparable to the human clinical condition (supra). These occlusions were done at the genu of the corpus callosum, at the main trunk proximal to this, or of individual anterior cerebral artery branches.

In 1934 (34), Watts reported that occlusion of the anterior cerebral arterial system in young or mature monkeys (*Macaca mulatta*) and one baboon (*Papio papio*) caused few if any noticeable neurological changes. These anterior cerebral artery occlusions were done at the genu of the corpus callosum. Watts speculated that the lack of any neurological dysfunction was probably attributable to compensation by vasculature from the intact middle cerebral arterial system. This implied conclusion is based upon his observation that Critchley's analysis (32) of human cases of anterior cerebral artery occlusion probably involved patients who also had compromised (reduced pressure) middle cerebral artery function as a result of reduced hypothalamic activity. Similarly, Watts (34) had found that in monkeys with hypothalamic

damage an anterior cerebral artery occlusion led to symptoms similar to those described by Critchley.

Unilateral Occlusion of the Middle Cerebral Artery

The majority of ischemic episodes in humans occur as a result of occlusion(s) of the middle cerebral artery, or one of its penetrating branches (31).

PRIMATES, DOGS, CATS In 1937, Peterson & Evans (35) reported the effects of permanent unilateral middle cerebral artery occlusion in the monkey. While it was observed that collateral circulation continues, the reduction in blood flow to the affected brain areas causes tissue necrosis (microscopic). Experiments in 1938, 1949, 1956 (36–38) reported that permanent middle cerebral artery occlusion in the dog leads to cerebral infarcts, accompanied by pronounced hemiparesis and other neurological defects. A temporary unilateral occlusion of the middle cerebral artery in the monkey for a minimum of 50 min (37) produced a pathology similar to that found with permanent middle cerebral artery occlusion. Rasmussen (37) reported that similar permanent occlusions of the anterior or posterior cerebral arteries did not produce infarcts. Using an "extradural" approach in cats (retro-orbital), Sundt et al and O'Brien (39, 40) found that middle cerebral artery occlusion (using Mayfield surgical clips) caused clear neurological deficits correlated with the production of cerebral infarcts of variable size. In the cat, using a transorbital approach, consistent infarct production occurs (although of variable size) with commensurate neurological deficits that are analogous to the human condition (41, 42). Identical sequelae using a transorbital approach in the baboon (*Papio cynocephalus*) were found (43).

RODENTS Since 1975 (44) use of unilateral occlusion of the rat middle cerebral artery has been reported. The advantages of using a small, inexpensive, behaviorally testable animal such as the rat are clear. The surgical procedure necessary to isolate and occlude the middle cerebral artery involve greater surgical precision, skill, and support equipment (e.g. fine dissecting surgical tools and a high-powered stereo-operating microscope). Robinson's (44) early studies indicated that the incidence of an infarct was variable. Infarct size was highly variable involving only the frontoparietal cortex, and did not extend subcortically. Behavioral long-term neurological dysfunction was found to be nominal except for a 2–3 wk period of hyperactivity. Later it was observed that the consistency of producing an infarct depends on the location of the occlusion on the middle cerebral artery. Tamura et al (45) reported that the closer the occlusion is to the origin (circle of Willis; below the olfactory tract) of the middle cerebral artery, the higher the incidence of a reproducible infarct. It was concluded that (45) the larger the rat, the greater

the necessity for occluding the artery near its origin. In male Sprague-Dawley rats greater than 350 gr, occlusions of the middle cerebral artery 1 mm below the rhinal fissure result in inconsistent infarcts, whereas in those weighing 250–325 grams there is high consistency of infarct production (C. G. Wakade, unpublished data). In a more detailed study (47), it was reported that not only the site but also the length of the occlusion on the middle cerebral artery, namely from the origin of the artery up to the rhinal fissure, defines its optimal capability to produce a large and consistent infarct. To achieve this optimal result the surgical procedure is considerably more invasive, involving retraction of the hemisphere to gain access to the middle cerebral artery's origin at the circle of Willis. In contrast, when the occlusion is done at or above the rhinal fissure, little or no neurological dysfunction is found, and infarct production (48, 49) is, if any, highly variable.

Detailed biochemical data has been reported as a result of unilateral middle cerebral artery occlusion (50, 51). Robinson has also reported that differences in hyperactivity and neurotransmitter changes are correlated with middle cerebral artery ligations that are restricted to the right hemisphere. These ligations were done above the rhinal fissure (52).

Occlusion of the Middle Cerebral Artery and Common Carotid Artery

The variability of infarct size and incidence that unilateral middle cerebral artery occlusion produces in the rat led Chen et al (49) to propose a modification of the middle cerebral artery model. This involves permanent ligation of the common carotid artery, followed first by permanent occlusion of the ipsilateral middle cerebral artery and then by a 1 hr temporary clamping of the contralateral common carotid artery. The model is based on the reasoning that the collateral circulation in the middle cerebral artery territory is decisive in infarct occurrence after middle cerebral artery ligation and that interruption would lead to a consistent and substantial occurrence of infarcts in the middle cerebral artery territory. In addition, the reduction in blood flow by the common carotid artery ligation and clamping result in hypotension, which is an underlying predisposition to ischemia. Slivka et al (53), using unilateral middle cerebral artery occlusion and ipsilateral common carotid artery ligation in the rabbit, report an inconsistent production of infarcts. However, when hypotension was induced by use of halothane, there was consistent infarct production (53). The modified middle cerebral artery rat occulsion model [middle cerebral artery occlusion plus common carotid artery occlusion] produces a *consistent, large, focal* ischemic lesion (49). The damage to the parietal area is large enough to justify assessment of functional losses and recovery. This *reproducibility of a localized infarct* in the middle cerebral artery occlusion plus common carotid artery occlusion model is advantageous

for biochemical and histological tissue analyses. The predictable, *progressive* spread of the ischemic area over time allows one to investigate whether treatment prevents the spread from occurring. Analyses can be carried out at the infarct site, and at peri-infarct loci (surrounding and remote areas from the primary infarct site).

In this model the common carotid arteries of the rat are isolated. A vertical 1.5 cm incision is made midway between the left orbit and external auditory canal. The skin is retracted, the temporalis muscle is separated and retracted. Using a saline cooled dental drill a 2 × 2 mm craniectomy is made 1mm rostral to the anterior junction of the zygoma and the squamosal bone. The dura is opened through a cruciate incision using a fine needle. The middle cerebral artery runs upward and is distinguished from its accompanying vein by being straighter and usually having fewer branches. The arachnoid on either side of the artery is divided by using a fine needle, and the artery is coagulated with bipolar radiofrequency applied through jewelers forceps 1 mm below the rhinal fissure and then at the point of the middle cerebral artery bifurcation distributing branches to frontal, parietal, and occipital regions (54–57). Following this cauterization, the artery is cut at the occlusion sites. The craniectomy is covered with gelfoam, soft tissues are allowed to fall back into place, and the skin sutured. Immediately preceeding the middle cerebral artery occlusion, the ipsilateral common carotid artery is permanently ligated. A pair of nontraumatic micro-aneurysm clips are applied to the contralateral common carotid artery for 60 min and then removed. Animals in which blood flow is not reestablished are discarded. Detailed analyses of edema, tissue ions, membrane ATPase are reported (54, 55). Further, behavioral tasks sensitive to this procedure have been studied in order to chart quantitative changes following the ischemic attack, thereby allowing for assessment of any therapeutic intervention. Specifically, it has been reported that the middle cerebral artery occlusion plus common carotid artery occlusion affects activity levels (creates hyperactivity), pole grasping and balancing, inclined board behavior, maze running, and cognitive function as measured in a operant-conditioned, nondiscriminatory reversal behavior (56, 57).

EMBOLIC STROKE: MULTIPLE ISCHEMIA

Six to 10% of all diagnosed stokes result from emboli. An ingenious model for this was reported first in 1955 where autologous blood clots were injected in animals (11, 58, 59). This procedure was again reported (60–62) using the rat. In 1970 (63) researchers used microfil orange polymer to create emboli that were injected into the common carotid artery of dogs. Recently more quantitative methods have been developed using calibrated microspheres as emboli. These models may be significant because of the recent national focus

on dementia that has motivated neuroscientists to extend research to include studies of an animal model for a significant class of dementia, Multiple Infarct Dementia. Vascular dementia is the second most frequent etiology of dementia. It is believed to result from numerous small infarcts and as a diagnosis has been designated Multi-Infarct Dementia (MID) (64–68). It has been hypothesized that a variety of vascular disorders and therefore mechanisms may give rise to MID. It has been treated as a single entity, although there are reports of two sub-classes: one minor group, where the infarcts are largely subcortical, and the larger group where the infarcts are primarily cortical (>85%) (68). Clinically, patients show dementia in combination with multiple behavioral and neurological dysfunctions (69).

A very recently developed method [1987–88] exists for producing multiple infarcts in small animals (79, 71). This involves injection of microspheres into the common carotid artery. The spheres used are small enough to penetrate terminal vessels. These spheres are evenly distributed within the penetrating arterioles where the resulting infarcts correspond to the distribution of the spheres. Infarcts of 200–500 um in diameter dominate. Too large spheres must be avoided as they will result in blockage of larger vessels and lead to a massive ischemic episode. There is a direct correlation between the number of spheres trapped in the brain and the extent of tissue damage and neurological deficit. When larger vessels are occluded this clear correspondence does not exist. The primary purpose of this approach has been to screen potential therapeutic treatments for stroke (70, 71). As a result, the number and size of the spheres injected have been such that they cause severe neurological damage and death. Smaller sphere size and number (dose) must be used and more discriminative functional assessments must be done in order to determine what sphere parameters are appropriate for an animal model where functional deficits occur, rather than relying on mortality alone.

Male Sprague-Dawley rats (300 gr) are anesthetized with halothane. The right common carotid artery is exposed and the external carotid and pterygopalatine arteries are ligated with No. 0 silk thread. The common carotid artery is cannulated with a plastic tube previously filled with heparinized saline. The cannula is then injected (0.5 ml gas-tight Hamilton syringe) with a suspension of the microspheres, followed by a flush of 0.5 ml saline. The common carotid artery is then permanently ligated. The polystyrene 15 um microspheres are prepared in 0.05% Tween-80 in normal saline followed by 5 min of full power sonication. A 100 ul aliquot is taken and immediately transferred to the gas-tight syringe (supra). A dose of 255 ugr of these spheres was found to induce an ES_{50} [effective dose of microspheres required to produce a clinically apparent stroke including death in 50% of the animals] (70, 71). Radiolabeled spheres can be used to quantitate the number of infarcts based upon the number of spheres that localize in the central nervous system.

CONCLUSION

Although there is no animal model identical to any of the predominant types of central nervous system stroke, each model offers characteristics that may be more or less advantageous for the study of the underlying pathophysiology of ischemia and its drug treatment. In every model it is clear that many variables are operative that can affect the resulting ischemic pathology. Caution is needed in controlling these variables, particularly since they can affect interpretation of therapeutic outcome. The spectrum of possible pharmacological treatments for stroke is now large. Choosing an appropriate model is therefore critical in attempting to demonstrate either efficacy or the mechanism(s) by which any therapeutic effect is observed. Although most stroke models are usable for pathological and biochemical analyses, the ability to quantitatively assess functional deficits and their amelioration may well be the most significant index of the efficacy of any therapy used for the treatment of brain pathology.

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